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Morphological and molecular identification of two new species of *Phlebiella* (Polyporales, Basidiomycota) from southern China

Tong-Kai Zong[1, 2], Chang-Lin Zhao[1, 2*]

[1] Key Laboratory for Forest Resources Conservation and Utilization in the Southwest Mountains of China, Ministry of Education, Southwest Forestry University, Kunming 650224, P.R. China

[2] College of Biodiversity Conservation, Southwest Forestry University, Kunming 650224, P.R. China

* Corresponding author: fungichanglinz@163.com

With 5 figures and 1 table

Abstract: Two new wood-inhabiting fungal species, *Phlebiella gossypina* and *P. wuliangshanensis* spp. nov., are proposed based on a combination of morphological features and molecular characters. *Phlebiella gossypina* is characterized by annual, resupinate, gossypine to byssaceous basidiomata, a monomitic hyphal system with clamped generative hyphae, heavily encrusted with large crystal among hyphae and subglobose to globose, thin-walled, warted basidiospores measuring 3.3–4.4 × 2.8–4 µm. *Phlebiella wuliangshanensis* is characterized by annual, resupinate basidiomata with pruinose to farinaceous to grandinoid hymenial surface, a monomitic hyphal system with clamped generative hyphae and subglobose, thin-walled, warted basidiospores (2.8–3.5 × 2.5–3 µm). Sequences of ITS gene regions of the studied samples were generated. The phylogenetic analysis based on molecular data of ITS sequences reveled that these two species are nested in the *Phlebiella* clade and supported the novelty of them.

Keywords: corticioid fungi; taxonomy; wood-rotting fungi; Yunnan province

Introduction

Phlebiella P. Karst. was typified by *P. vaga* (Fr.) P. Karst. (Karsten 1890), which is a genus characterized by a combination of resupinate to effused basidiomata with a ceraceous to subgelatinous consistencey, hymenophore smooth to porulose to reciculate to grandinioid, a monomitic hyphal structure with clamped generative hyphae, basidia pleural and basidiospores hyaline, thin to thick-walled, warted, subgloboseto ellipsoid to cylindrical (Karsten 1890, Bernicchia & Gorjón 2010). So far about 10 species have been accepted

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in the genus worldwide (Karsten 1890, Bondartsev 1953, Hjortstam & Larsson 1987, Roberts 1995, Bernicchia & Gorjón 2010).

Wood-rotting fungi are a cosmopolitan group and they have a rich diversity on the basis of growing on boreal, temperate, subtropical, and tropical vegetations (Gilbertson & Ryvarden 1986, Núñez & Ryvarden 2001, Bernicchia & Gorjón 2010, Dai 2012, Ryvarden & Melo 2014, Dai et al. 2015). During investigations on wood-inhabiting fungi in southern China, two additional taxa of *Phlebiella* were found which could not be assigned to any described species. In this study, the authors expand samplings to examine morphological characters and molecular phylogenetic evidence of new species within *Phlebiella*.

Materials and methods

Morphological studies

The studied specimens are deposited at the herbarium of Southwest Forestry University (SWFC), Kunming, Yunnan Province, P.R. China. Macro-morphological descriptions are based on field notes. Special colour terms follow Petersen (1996). Micro-morphological data were obtained from the dried specimens, and observed under a light microscope following Dai (2012). The following abbreviations were used: KOH = 5% potassium hydroxide, CB = Cotton Blue, CB– = acyanophilous, IKI = Melzer's reagent, IKI– = both inamyloid and indextrinoid, L = mean spore length (arithmetic average of all spores), W = mean spore width (arithmetic average of all spores), Q = variation in the L/W ratios between the specimens studied, n (a/b) = number of spores (a) measured from given number (b) of specimens.

Molecular procedures and phylogenetic analyses

A CTAB rapid plant genome extraction kit-DN14 (Aidlab Biotechnologies Co., Ltd, Beijing) was used to obtain genomic DNA from dried specimens, according to the manufacturer's instructions that were slightly modified by grinding a small piece of dried fungal specimen (about 30 mg) to powder with liquid nitrogen. The powder was transferred to a 1.5 mL centrifuge tube, suspended in 0.4 mL of lysis buffer, and incubated in a 65 °C water bath for 60 min. After that, 0.4 mL phenol-chloroform (24:1) was added to each tube and the suspension was shaken vigorously. After centrifugation at 13,000 rpm for 5 min, 0.3 mL supernatant was transferred to a new tube and mixed with 0.45 mL binding buffer. The mixture was then transferred to an Adsorbing Column (AC) for centrifugation at 13, 000 rpm for 0.5 min. Then, 0.5 mL inhibitor removal fluid was added in the AC for a centrifugation at 12,000 rpm for 0.5 min. After washing twice with 0.5 mL washing buffer, the AC was transferred to a clean centrifuge tube, and 100 mL elution buffer was added to the middle of the adsorbed film to elute the genomic DNA. The internal transcribed spacer region (ITS) was amplified with primer pair ITS5 and ITS4 (White et al.

1990). The PCR procedure for ITS was as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 40 s, 58 °C for 45 s, and 72 °C for 1 min; and a final extension of 72 °C for 10 min. The PCR products were purified and directly sequenced at the Kunming Tsingke Biological Technology Limited Company, Kunming, Yunnan Province P.R. China. All newly generated sequences were deposited in GenBank (Table 1).

Sequencher 4.6 (GeneCodes, Ann Arbor, MI, USA) was used to edit the DNA sequence. Sequences were aligned in MAFFT 7 (http://mafft.cbrc.jp/alignment/server/) using the "G-INS-I" strategy and manually adjusted in BioEdit (Hall 1999). The sequence alignment was deposited in TreeBase (submission ID 27656). Sequences of *Dacrymyces stillatus* Nees and *Dacryopinax spathularia* (Schwein.) G.W. Martin retrieved from GenBank were used as an outgroup in the ITS analysis (Fig. 1) following He et al. (2019).

Maximum parsimony analysis was applied to the ITS dataset sequences. Approaches to phylogenetic analysis followed Zhao & Wu (2017), and the tree construction was performed in PAUP* version 4.0b10 (Swofford 2002). All characters were equally weighted and gaps were treated as missing data. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Max-trees were set to 5000, branches of zero length were collapsed and all parsimonious trees were saved. Clade robustness was assessed using a bootstrap (BT) analysis with 1,000 replicates

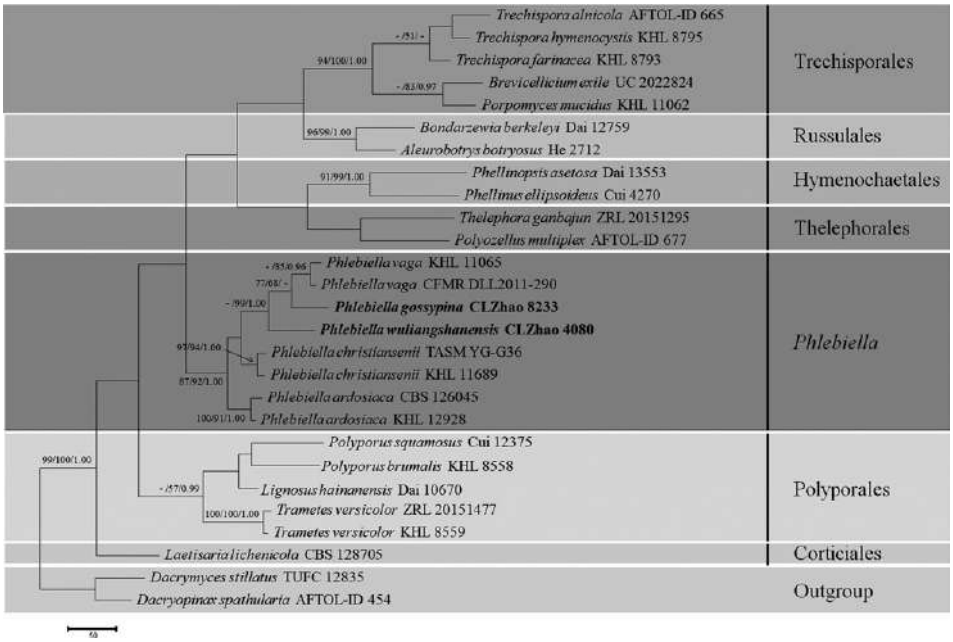

Fig. 1. Maximum Parsimony strict consensus tree illustrating the phylogeny of two new species of *Phlebiella* and related order based on ITS sequences. Branches are labeled with maximum likelihood bootstrap values higher than 70%, parsimony bootstrap values higher than 50% and Bayesian posterior probabilities more than 0.95.

Table 1. List of species, specimens and GenBank accession numbers of sequences used in this study.

Species name	Sample no.	GenBank accession no. ITS	References
Aleurobotrys botryosus	He 2712	KX306877	Dai & He 2019
Bondarzewia berkeleyi	Dai 12759	KJ583202	Chen et al. 2016
Brevicellicium exile	UC 2022824	KP814539	Rosenthal et al. 2017
Dacrymyces stillatus	TUFC 12835	AB712464	Shirouzu et al. 2013
Dacryopinax spathularia	AFTOL-ID 454	AY854070	He et al. 2019
Laetisaria lichenicola	CBS 128705	MH864964	Vu et al. 2019
Lignosus hainanensis	Dai 10670	NR154112	Cui et al. 2011
Phellinopsis asetosa	Dai 13553	KJ425524	Zhou 2015
Phellinus ellipsoideus	Cui 4270	JQ837948	Cui & Decock 2013
Phlebiella aff. ardosiaca	KHL 12928	EU118658	Larsson 2007
P. ardosiaca	CBS 126045	MH864060	Vu et al. 2019
P. christiansenii	TASM YG-G36	MT526342	Gafforov et al. 2020
P. christiansenii	KHL 11689	EU118659	Larsson 2007
P. gossypina	CLZhao 8233	MW545957	This study
P. gossypina	CLZhao 4149	MW545958	This study
P. gossypina	CLZhao 4316	MW545959	This study
P. gossypina	CLZhao 8370	MW545960	This study
P. gossypina	CLZhao 8398	MW545961	This study
P. vaga	KHL 11065	EU118660	Larsson 2007
P. vaga	CFMR DLL2011-290	KJ140766	Brazee et al. 2014
P. wuliangshanensis	CLZhao 4080	MW545962	This study
P. wuliangshanensis	CLZhao 4308	MW545963	This study
Polyozellus multiplex	AFTOL-ID 677	DQ411528	He et al. 2019
Polyporus brumalis	KHL 8558	AF347108	Larsson 2004
P. squamosus	Cui 12375	KX851641	He et al. 2019
Porpomyces mucidus	KHL 11062	AF347091	Larsson 2007
Thelephora ganbajun	ZRL 20151295	LT716082	Zhao et al. 2017
Trametes versicolor	ZRL 20151477	LT716079	Zhao et al. 2017
T. versicolor	KHL 8559	AF347107	Larsson 2004
Trechispora alnicola	AFTOL-ID 665	DQ411529	He et al. 2019
T. farinacea	KHL 8793	AF347089	Larsson et al. 2004
T. hymenocystis	KHL 8795	AF347090	Larsson et al. 2004

(Felsenstein 1985). Descriptive tree statistics tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were calculated for each Maximum Parsimonious Tree generated. Sequences were also analyzed using Maximum Likelihood (ML) with RAxML-HPC2 through the Cipres Science Gateway (www.phylo.org; Miller et al. 2009). Branch support (BS) for ML analysis was determined by 1000 bootstrap replicates.

MrModeltest 2.3 (Nylander 2004) was used to determine the best-fit evolution model for each data set for Bayesian Inference (BI). BI was calculated with MrBayes 3.2.7a with a general time reversible (GTR+I+G) model of DNA substitution and a gamma distribution rate variation across sites (Ronquist et al. 2012). Four Markov chains were run for 2 runs from random starting trees, for 500,000 generations (Fig. 1) and trees were sampled every 100 generations. The first one-fourth generations were discarded as burn-in. A majority rule consensus tree of all remaining trees was calculated. Branches were considered as significantly supported if they received maximum likelihood bootstrap (BS) >75%, maximum parsimony bootstrap (BT) >75%, or Bayesian posterior probabilities (BPP) >0.95.

Results

The ITS dataset (Fig. 1) included sequences from 27 fungal specimens representing 24 species between *Phlebiella* clade and related order. The dataset had an aligned length of 1003 characters, of which 366 characters were constant, 203 were variable and parsimony-uninformative, and 434 were parsimony-informative. Maximum parsimony analysis yielded 6 equally parsimonious trees (TL = 2405, CI = 0.5002, HI = 0.4998, RI = 0.3450, RC = 0.1726). the best model for the ITS dataset estimated and applied in the Bayesian analysis: was GTR+I+G, lset nst = 6, rates = invgamma; prset statefreqpr = dirichlet (1,1,1,1). Bayesian analysis and ML analysis resulted in a similar topology as MP analysis, with an average standard deviation of split frequencies = 0.009879 (BI).

The phylogenetic tree (Fig. 1) inferred from ITS sequences revealed that the two new taxa clustered into *Phlebiella* clade and then grouped with *P. vaga* (Fr.) P. Karst.

Taxonomy

Phlebiella gossypina C.L. Zhao, sp. nov. Figs. 2, 3

MycoBank no.: MB 839014

Diagnosis: The species is characterized by its cotton to flocculent basidiomata with cream to buff hymenophore, a monomitic hyphal system with clamped generative hyphae and basidiospores subglobose, hyaline, thin-walled, warted, 3.3–4.4 × 2.8–4 µm.

Holotypus: CHINA. Yunnan Prov., Yuxi, Xinping County, Tea Horse Ancient Road Forestry Park, on the angiosperm trunk, 21 August 2018, CLZhao 8233 (SWFC), Genbank MW545957 (ITS).

Etymology: *Gossypina* (Lat.): referring to the gossypine hymenial surface.

Fruiting body: Basidiomata annual, resupinate, thin, without odor or taste when fresh, becoming membranous when fresh, gossypine to byssaceous upon drying, up to 8 cm long, 100–200 μm thick. Hymenial surface cream when fresh, cream to buff upon drying. Sterile indistinct, cream to buff.

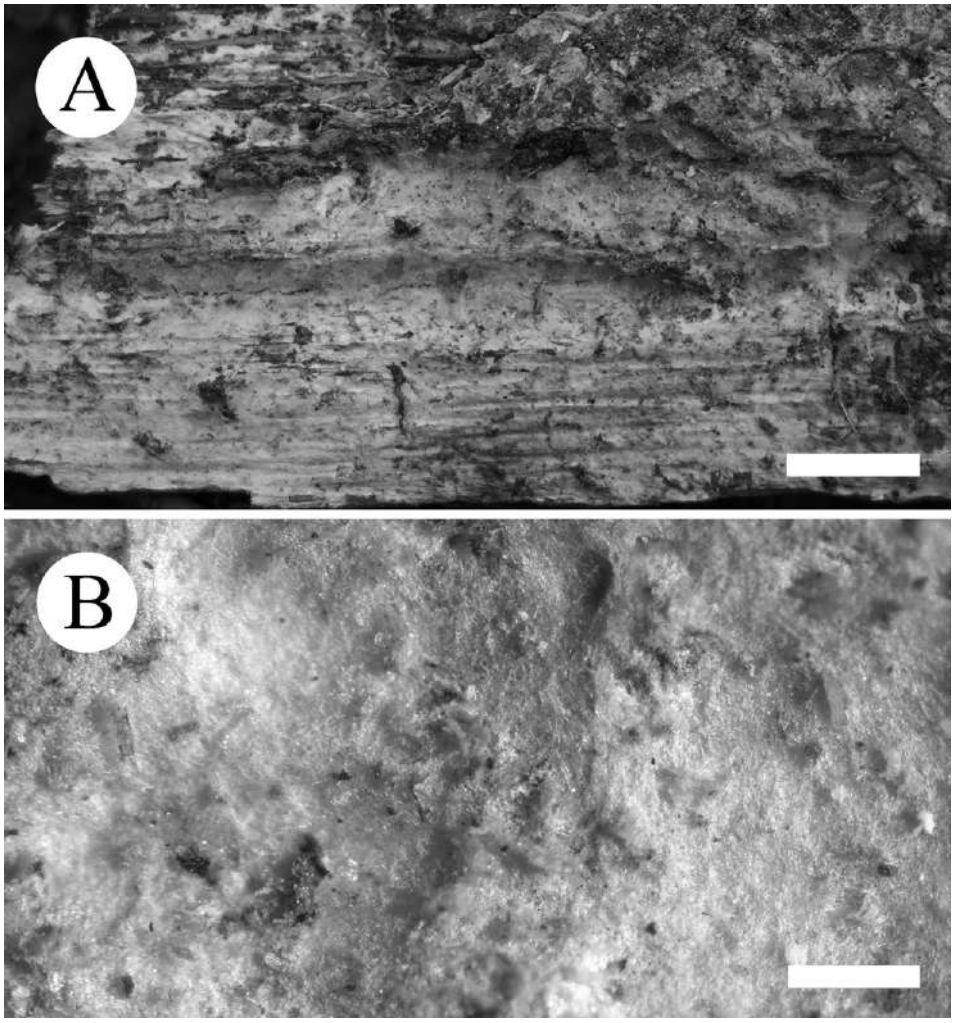

Fig. 2. A basidiomata of *Phlebiella gossypina* (holotype). Bar: A = 1 cm; B = 0.4 mm.

Hyphal structure: Hyphal system monomitic; generative hyphae with clamps, thin-walled, branched, 2.5–6 µm in diam., IKI–, CB–, tissues unchanged in KOH; presence of larger crystal among hyphae.

Hymenium: Cystidia and cystidioles absent; basidia pleural, barrel-shaped, with 4 sterigmata and a basal clamp, 14–23.5 × 4–7 µm; basidioles dominant, in shape similar to basidia, but slightly smaller.

Spores: Basidiospores subglobose to globose, hyaline, thin-walled, warted, IKI–, CB–, (3–)3.3–4.4(–4.6) × (2.5–)2.8–4(–4.3) µm, L = 3.84 µm, W = 3.34 µm, Q = 1.13–1.18 (n = 300/5).

Rot type: A white rot.

Additional specimens (paratypes) examined: CHINA. Yunnan Prov., Puer, Jingdong County, Wuliangshan National Nature Reserve, on the angiosperm trunk, 5 October 2017, CLZhao 4149, CLZhao 4316; Taizhong Town, on the angiosperm trunk, 23 August 2018, CLZhao 8370, CLZhao 8398 (SWFC).

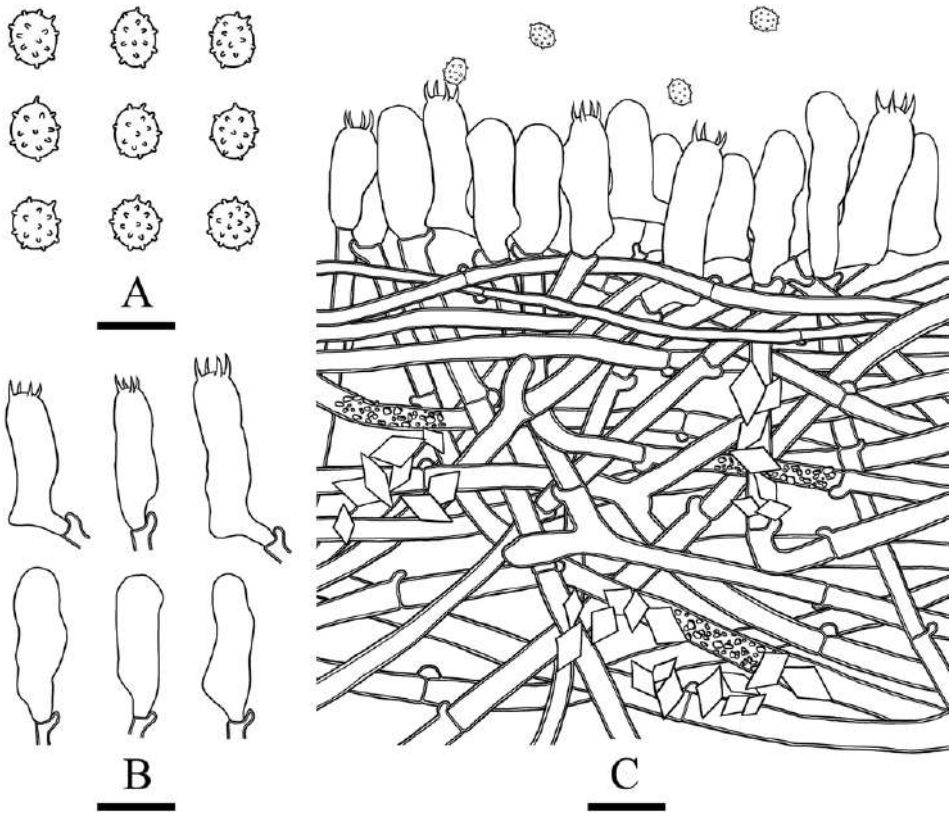

Fig. 3. Microscopic structures of *Phlebiella gossypina* (drawn from the holotype). A. Basidiospores; B. Basidia and basidioles; C. A section of basidiocarp. Bars: A = 5 µm; B, C = 10 µm.

Phlebiella wuliangshanensis C.L. Zhao, sp. nov. Figs. 4, 5

MycoBank no.: MB 839015

Diagnosis: The species is characterized by its membranaceous to farinaceous basidiomata with clay-pink to saffron hymenophore, a monomitic hyphal system with clamped generative hyphae and basidiospores subglobose, hyaline, thin-walled, warted, measuring as 2.8–3.5 × (2.3–)2.5–3 μm.

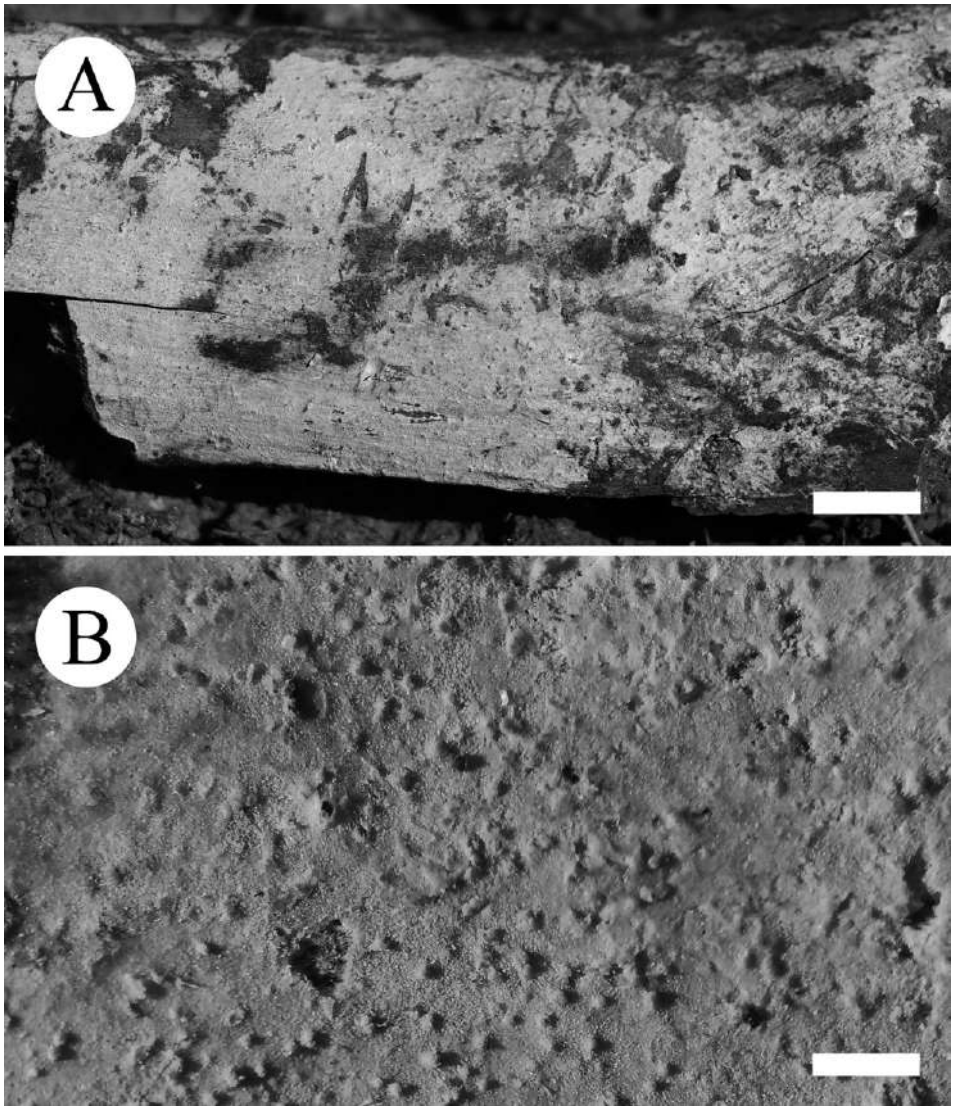

Fig. 4. A basidiomata of *Phlebiella wuliangshanensis* (holotype). Bar: A = 1 cm; B = 0.6 mm.

Holotypus: CHINA. Yunnan Prov., Puer, Jingdong County, Wuliangshan National Nature Reserve, on the angiosperm trunk, 5 October 2017, CLZhao 4080 (SWFC), Genbank MW545962 (ITS).

Etymology: *Wuliangshanensis* (Lat.): referring to the locality (Wuliangshan) of the type specimen.

Fruiting body: Basidiomata annual, resupinate, very difficult to separate from substrate, becoming ceraceous to leather when fresh, pruinose to farinaceous to grandinoid upon drying, up to 10 cm long, 150–300 µm thick. Hymenial surface smooth to tuberculate, flesh-pink to clay-pink when fresh, clay-pink to safforn upon drying. Sterile indistinct, clay-pink.

Hyphal structure: Hyphal system monomitic; generative hyphae with clamps, thin-walled, unbranched, 1.5–3.5 µm in diam., IKI–, CB–, tissues unchanged in KOH.

Hymenium: Cystidia and cystidioles absent; basidia pleural, clavate to cylindrical, with 4 sterigmata and a basal clamp, 12–18.5 × 3.5–5.5 µm; basidioles dominant, in shape similar to basidia, but slightly smaller.

Spores: Basidiospores subglobose, hyaline, thin-walled, warted, IKI–, CB–, (2.5–)2.8–3.5(–4) × (2.3–)2.5–3(–3.3) µm, L = 3.22 µm, W = 2.85 µm, Q = 1.14–1.19 (n = 60/2).

Rot type: A white rot.

Additional specimen (paratype) examined: CHINA. Yunnan Prov., Puer, Jingdong County, Wuliangshan National Nature Reserve, on the angiosperm trunk, 5 October 2017, CLZhao 4308 (SWFC).

Discussion

In the present study, two new species, *Phlebiella gossypina* and *P. wuliangshanensis* spp. nov., are described based on phylogenetic analyses and morphological characters.

Phylogenetically, Larsson (2007) focused on re-thinking the classification of corticioid fungi inferred from 5.8S and nLSU rDNA sequences to concern the higher order classification of basidiomycetes down to order, in which *Phlebiella ardosiaca* (Bourdot & Galzin) K.H. Larss. & Hjortstam, *P. christiansenii* (Parmasto) K.H. Larss. & Hjortstam and *P. vaga* nested in *Phlebiella* clade and then grouped with the orders of Corticiales, Polyporales and Trechisporales. In the present study, *P. gossypina* and *P. wuliangshanensis* are nested in the *Phlebiella* clade based on ITS sequence data (Fig. 1) and are grouped with *P. vaga*. But morphologically *P. vaga* differs from *P. gossypina* and *P. wuliangshanensis* by having the larger basidiospores (5–5.5 × 4–4.5 µm, Karsten 1890); in addition, the generative hyphae of *P. vaga* turn vinaceous red in KOH.

Morphologically, *Phlebiella gossypina* is similar to *P. ardosiaca*, *P. fibrillosa* (Hallenb.) K.H. Larss. & Hjortstam, *P. subflavidogrisea* (Litsch.) Oberw., based on the porulose to

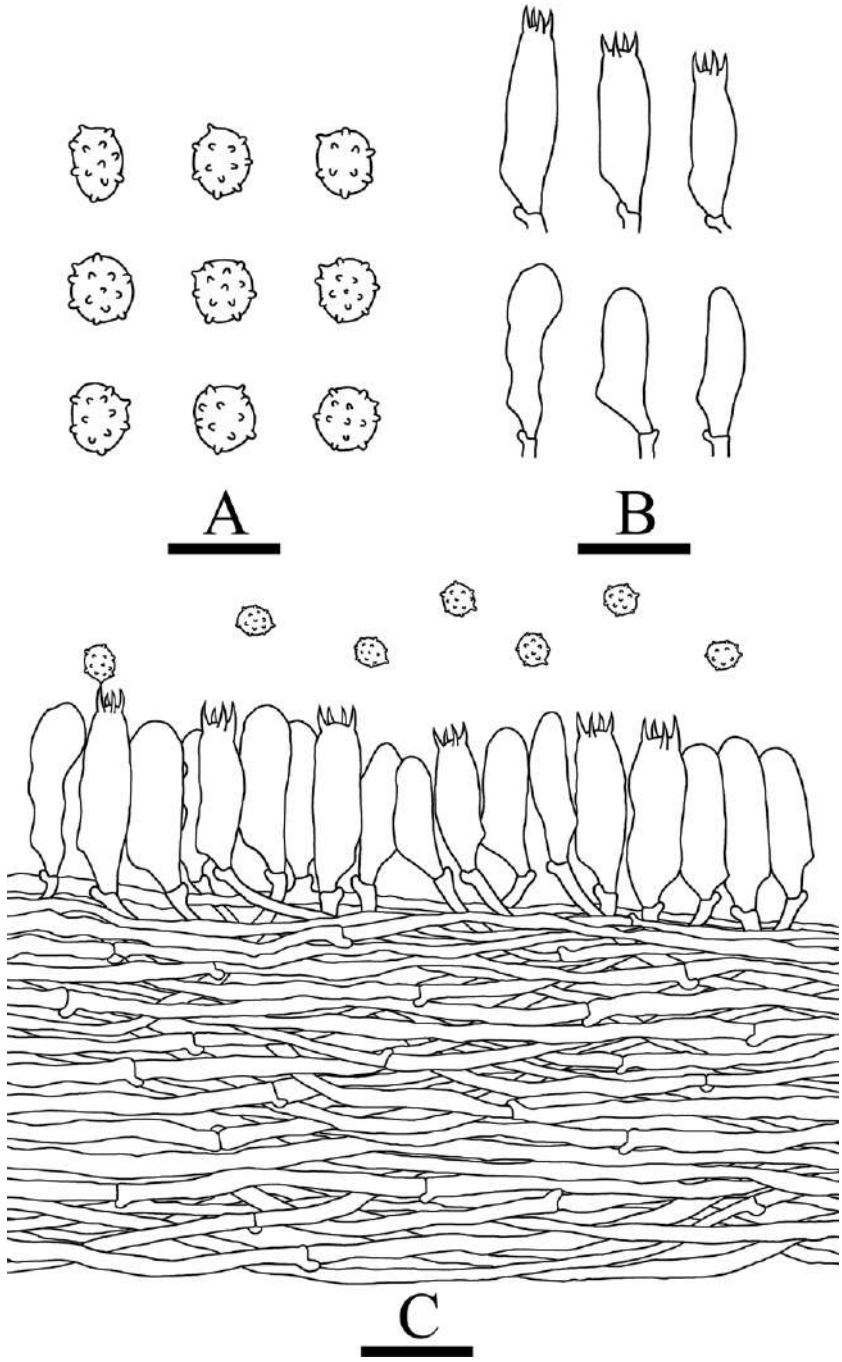

Fig. 5. Microscopic structures of *Phlebiella wuliangshanensis* (drawn from the holotype). A. Basidiospores; B. Basidia and basidioles; C. A section of basidiocarp. Bars: A = 5 µm; B, C = 10 µm.

gossypine hymenophore. However, *P. ardosiaca* differs from *P. gossypina* by having both larger and thick-walled basidiospores (5–6 × 5–5.5 µm, Hjortstam & Larsson 1987, Bernicchia & Gorjón 2010). *Phlebiella fibrillosa* can be distinguished by white to pale yellowish hymenial surface, presence of fibrillose with hyphal strands and the encrusted generative hyphae (Bernicchia & Gorjón 2010). *Phlebiella subflavidogrisea* differs in having the white to grayish hymenial surface, turning dark reddish brown in KOH and narrowly ellipsoid basidiospores (3.5–4.5 × 2–2.5 µm, Bernicchia & Gorjón 2010).

Phlebiella wuliangshanensis is similar to *P. californica* (Liberta) K.H. Larss. & Hjortstam, *P. christiansenii*, *P. gaspesica* (Liberta) K.H. Larss. & Hjortstam, based on the pruinose to farinaceous hymenophore, but *P. californica* can be distinguished by the bluish-gray hymenial surface and larger basidiospores (5.5–7 × 3–4 µm, Hjortstam & Larsson 1987). *Phlebiella christiansenii* differs from *P. wuliangshanensis* by having the effused basidiocarps with radially arranged rhizomorphs and larger basidiospores (6–7 × 4–4.5 µm, Bernicchia & Gorjón 2010). *Phlebiella gaspesica* differs in its effused basidiomata and cylindrical basidiospores (5.5–7 × 1.5–2 µm, Hjortstam & Larsson 1987).

In geographical distribution, five species of *Phlebiella* were reported from this region, but all of them were transferred to other genera (Dai 2011). The diversity of *Phlebiella* in China is still not well known, especially in the subtropical and tropical regions and many recently described taxa of wood-rotting fungi were from these areas (Cui & Dai 2006, Cui 2009, Yuan 2013, Chen et al. 2015, Zhao et al. 2015, Zhao et al. 2016, Zhao et al. 2019, Yuan et al. 2016, Zhao & Wu 2017, Shen et al. 2018). *Phlebiella gossypina* and *P. wuliangshanensis*, are also from the subtropics. It is possible that new taxa will be found after further investigations.

Acknowledgments

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